

Incidence and Pathogenic Effect of *Streptococcus pseudopneumoniae*

Colette Harf-Monteil,* Carole Granello, Cécile Le Brun, Henri Monteil, and Philippe Riegel

Institute of Bacteriology, UPRES-EA 3432, Louis Pasteur University, Strasbourg University Hospital, Strasbourg, France

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We evaluated the incidence of *Streptococcus pseudopneumoniae* in clinical isolates by phenotypic methods and DNA-DNA hybridization. The pathogenic role of this organism was investigated with the mouse peritonitis/sepsis model. Our results show a low incidence (1/120 pneumococcal isolates) and a potential pathogenic effect for *S. pseudopneumoniae*.

Streptococcus pneumoniae, a major cause of morbidity and mortality worldwide, remains a significant health threat. Correct identification of pneumococci from clinical samples and differentiation from other oral streptococci are essential for appropriate diagnosis and treatment. A novel species of viridans group streptococci resembling *S. pneumoniae* was recently described as *Streptococcus pseudopneumoniae* (1); phenotypic identification is based on susceptibility to optochin (OPT) in ambient atmosphere and OPT resistance in a 5% CO₂ atmosphere, bile salt insolubility, and the absence of pneumococcal capsule. Genotypic identification is based on DNA-DNA hybridization, whereas *sodA* sequencing and 16S rRNA gene sequencing are not discriminating. While the new species tested positive for the pneumolysin gene (*ply*), its pathogenic role was not investigated. In the present study, we assessed the clinical significance of this new species by examining its incidence among pneumococci from clinical samples and investigating its virulence in a mouse peritonitis/sepsis model system.

In total, 120 isolates presumptively identified as *S. pneumoniae* on the basis of Gram stain, catalase activity, and OPT susceptibility in ambient atmosphere were obtained from consecutive clinical samples of patients admitted to University Hospital, Strasbourg, France, during the period October 2004 to April 2005. Single pneumococcal isolates/patients/infections were analyzed. Eighteen isolates were collected from the lower respiratory tract, 57 were collected from the upper respiratory tract, 24 were collected from blood, 1 was collected from cerebrospinal fluid, 3 were collected from pleural fluid, and 12 were collected from other sterile body sites. Controls (*S. pseudopneumoniae* CCUG 49455^T and *S. pneumoniae* CCUG 28588^T) were included in all assays. Additional clinical strains (*S. pseudopneumoniae* CCUG 48465, CCUG 50866, CCUG 50867, CCUG 50868, CCUG 50869, CCUG 50870, and CCUG 50871) described by Arbique et al. (1) were included in virulence assays.

OPT susceptibility testing was performed by the disk diffusion method with a 6-mm disk (Bio-Rad) on sheep blood agar (Trypticase soy agar [bioMérieux] supplemented with 5% sheep blood); plates were incubated for 18 to 24 h at 35°C in

ambient air and in a 5% CO₂ atmosphere. Solubility in bile salt (sodium deoxycholate [Merck]) was determined in tubes (4). Capsules were detected by observing a halo around pneumococci with India ink at a ×100 to ×400 magnification. Capsular agglutination tests were performed by the Pastorex test (Bio-Rad) according to the instructions of the manufacturer.

DNA-DNA hybridization was performed as described previously (8). Hybridization between labeled DNA and the fragmented DNA preparation was carried out at 60°C for 16 h in 0.42 M NaCl by the nuclease-trichloroacetic acid method (3). The values of DNA-DNA relatedness given are the means of the results of three independent experiments. Detection of *ply* in the extracted DNA was performed by PCR with primers specific for a 170-bp region, as described previously (1, 9). The PCR products were analyzed by electrophoresis on 2% agarose gels, with visualization of the amplicon with ethidium bromide.

Immunocompetent 6-week-old Swiss mice were infected by intraperitoneal injection (0.5 ml) of an exponential-phase culture in brain heart infusion broth (Bio-Rad) (10⁸ CFU per mouse) (2) and observed for a 7-day period. Controls received identical injections of broth. The experiments were performed in triplicate for each isolate and were repeated twice. Dead and sacrificed animals were examined; bacterial cultures of peritoneal fluid and spleen sections were performed on sheep blood agar, and spleen section imprints were Gram stained.

All 120 isolates were OPT susceptible in ambient atmosphere. Six strains (5%) were OPT resistant (zone size, 6 mm) when incubated in CO₂. Among the six OPT-resistant isolates, three were capsular agglutination test negative, and two showed no capsule with India ink. All isolates were soluble in bile salt, except one (IBS-370), which was identified as *S. pseudopneumoniae* (OPT resistant in CO₂, insoluble in bile salt, capsular agglutination negative, nonencapsulated).

DNA-DNA hybridization assays performed between strain IBS-370 and the type strains *S. pneumoniae* CCUG 28588 and *S. pseudopneumoniae* CCUG 49455 yielded values of 47% and 90%, respectively (Table 1). DNA-DNA similarities of the other five OPT-resistant isolates were low (<50%) for *S. pseudopneumoniae* and varied for *S. pneumoniae*, being high (82%) for one *S. pneumoniae* isolate and low for the other (two unidentified isolates will need further study, and two isolates were identified phenotypically as *S. constellatus* and *S. parasanguis*). Both type strains as well as IBS-370 tested positive for *ply* (Table 1). Virulence assays resulted in 100% dead mice for *S. pneumoniae* CCUG 28588^T and *S. pseudopneumoniae*

* Corresponding author. Mailing address: Institute of Bacteriology, Louis Pasteur University, Strasbourg University Hospital, 3 rue Koeberle, 67000 Strasbourg, France. Phone: 33 3 90 24 37 62. Fax: 33 3 88 25 11 13. E-mail: colette.harf-monteil@medecine.u-strasbg.fr.

TABLE 1. Genotypic and virulence test results^a

Strain	% DNA-DNA hybridization with:		Virulence in mice			
	<i>S. pneumoniae</i> CCUG 28588 ^T	<i>S. pseudopneumoniae</i> CCUG 49455 ^T	Death/survival status ^b	Result for spleen samples		Result for peritoneal swabbing
				Imprints	Swabbing	
<i>S. pneumoniae</i> CCUG 28588 ^T	100	48	D 24	+	+	+
<i>S. pseudopneumoniae</i> CCUG 49455 ^T	37	100	S	–	–	–
<i>S. pseudopneumoniae</i> IBS-370	47	90	D 36	+	+	+
Additional <i>S. pseudopneumoniae</i> clinical strains ^c			D 36	+	+	+

^a All strains had the *ply* gene.

^b D 24, death after 24 h; S, survival; D 36, death after 36 h.

^c CCUG 48465, CCUG 50866, CCUG 50867, CCUG 50868, CCUG 50869, CCUG 50870, and CCUG 50871, described by Arbique et al. (1).

CCUG 48465, CCUG 50866, CCUG 50867, CCUG 50868, CCUG 50869, CCUG 50870, CCUG 50871, and IBS-370 after 24 h and 36 h, respectively; all cultures and spleen imprints were positive (Table 1). Strain IBS-370 was isolated from bronchoalveolar lavage fluid in a 62-year-old inpatient suffering from interstitial bilateral syndrome who formerly suffered from right-sided axillary pneumococcal pleuropneumonia. All mice survived after *S. pseudopneumoniae* CCUG 49455^T injection; all cultures and imprints were negative.

Phenotypic and genotypic results clearly identified strain IBS-370 as *S. pseudopneumoniae*. This new species proved to be seldom represented (1 of 120 *S. pneumoniae* isolates collected in our hospital over 7 months). Despite their lack of capsule, *S. pseudopneumoniae* IBS-370, CCUG 48465, CCUG 50866, CCUG 50867, CCUG 50868, CCUG 50869, CCUG 50870, and CCUG 50871 were clearly pathogenic, killing 100% of infected animals in 36 h. The increased virulence of *S. pneumoniae* CCUG 28588^T (lethal effect in 24 h) might be assigned to the capsule, which protects pneumococci from engulfment and digestion by macrophages and polymorphonuclear leukocytes during the host early immune response. Among the virulence factors required for invasive disease in *S. pneumoniae*, the lethal effect stems from the production of pneumolysin, the most broadly potent virulence factor (2, 6). The *ply* gene was shown to also be present in phylogenetically related species by horizontal transfer of genes, suggesting a pathogenic role (1, 5). All *S. pseudopneumoniae* strains harbored the *ply* gene. *S. pseudopneumoniae* IBS-370, as well as all additional clinical strains, was clearly pathogenic, while *S. pseudopneumoniae* CCUG 49455^T showed no virulence, suggesting a lack of pneumolysin production or a lack of virulence cofactors which remain to be determined.

In our results, OPT-resistant streptococci in a CO₂ atmosphere included pathogens such as *S. pseudopneumoniae* and *S. pneumoniae*. OPT resistance in *S. pneumoniae* is uncommon but leads to problems in identification (1, 7). Thus, for correct diagnosis and treatment of lower respiratory tract infections, OPT susceptibility testing should be performed in the ambient atmosphere rather than in CO₂ to avoid exclusion of OPT-

resistant *S. pneumoniae* and underestimation of pathogenic *S. pseudopneumoniae*. In addition, phenotypic identification can be completed by OPT susceptibility testing in CO₂ and bile salt solubility testing. Genotypic identification by DNA-DNA hybridization can be performed if necessary.

In conclusion, our results highlight a low incidence and potential pathogenic role of *S. pseudopneumoniae*. Furthermore, they underscore the complexity of oral streptococci regarding the relationship between their identification and the virulence factors produced. This may lead to laboratories taking account of oral streptococci isolated from specimens of patients suffering from severe lower respiratory tract infections.

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